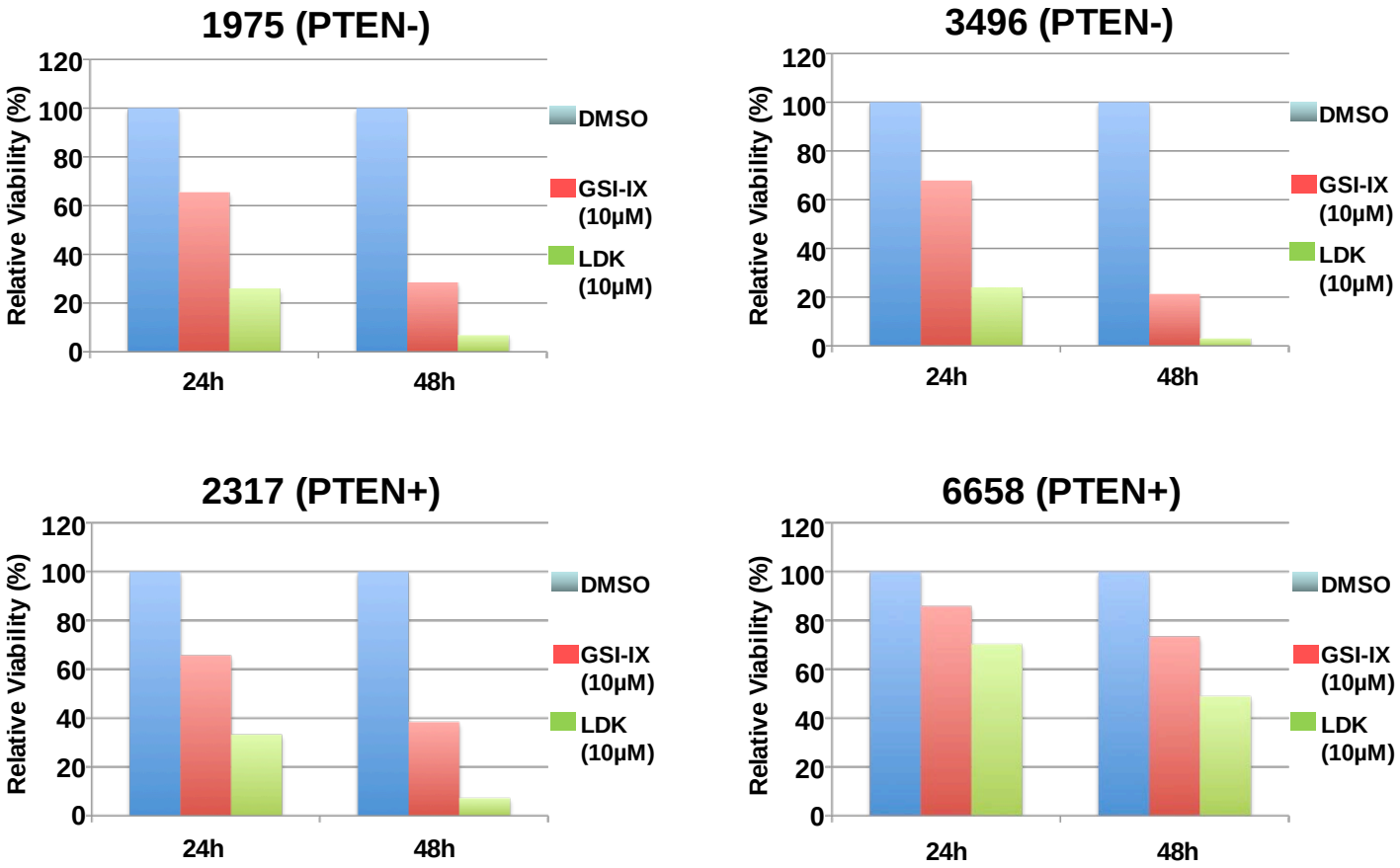
Supplemental Figure 1. LDK decreases viability of primary murine T-ALL cells. Four murine samples derived from primary T-ALL arising in *Atm*$^{-/-}$ mice and expressing high levels of PEST-truncated activated NOTCH1 were evaluated for growth inhibition in response to Lenaldekar (LDK). Two T-ALL cell lines are PTEN- (1975, 3496) and two are PTEN+ (2317, 6658). Cells were cultured for 24 or 48 hours with Interleukin-7 (10ng/ml) and treated with DMSO vehicle, gamma-secretase inhibitor IX (GSI-IX) or LDK (10μM). After 24 and 48 hours, cell viability was quantified using the Cell Titre Blue Assay and expressed as percent relative to vehicle treatment at each time point. Experiment was repeated, one representative experiment is shown.



Supplemental Figure 2. LDK has selective activity against hematological malignancies and induces apoptosis in Jurkat cells. (A) LDK was submitted to the NCI for dose-response testing on the NCI60 panel of cancer cell lines. Results are shown as the IC_{50} for each cell line (Red = high sensitivity/low IC_{50}, Yellow/orange = medium sensitivity/medium IC_{50}, Green = Low sensitivity/high IC_{50}). Results are organized by cancer cell type with names of cell lines tested shown below their IC_{50} listings in the same order. (B) Annexin V-FITC/Propidium Iodide staining of Jurkat cells incubated for 24 hours with DMSO, 1µM LDK, or 5µM Camptothecin. (C) Caspase activation demonstrated by PARP cleavage (arrow) by 8 hours of incubation in 1µM LDK. (D = DMSO vehicle. Vinculin loading control. Non-contiguous sections of same gel). (D) LDK induction of apoptosis demonstrated by activated Caspase-3 immunostaining of Jurkat cells incubated for 16 hrs in DMSO, 1µM LDK, or 5µM Camptothecin. Scale bars = 2µm. For panel C, Western blots were scanned at room temperature using the Epson Expression 1680 scanner and software (Long Beach, CA), 16-bit grayscale acquisition, 600 dpi resolution. Image processing was done utilizing Adobe Photoshop software v9.0.2 (San Jose, CA). For panel D, fluorescence microscope used was Nikon Eclipse E600 (Melville, NY) using Nikon Plan Fluor 40X objective lens (Melville, NY) at room temperature. Fluorochromes used were DAPI and Alexa Fluor 568 goat anti-rabbit IgG secondary antibody (Invitrogen, Grand Island, NY). Camera used was Nuance CRI model N-MSI-420-FL (Hopkinton, MA). Image capture was done using IP Lab software version 4.0 (Becton, Dickinson, Sparks, MD), with image presentation processed using Image J version 1.43U (NIH, Bethesda, MD).

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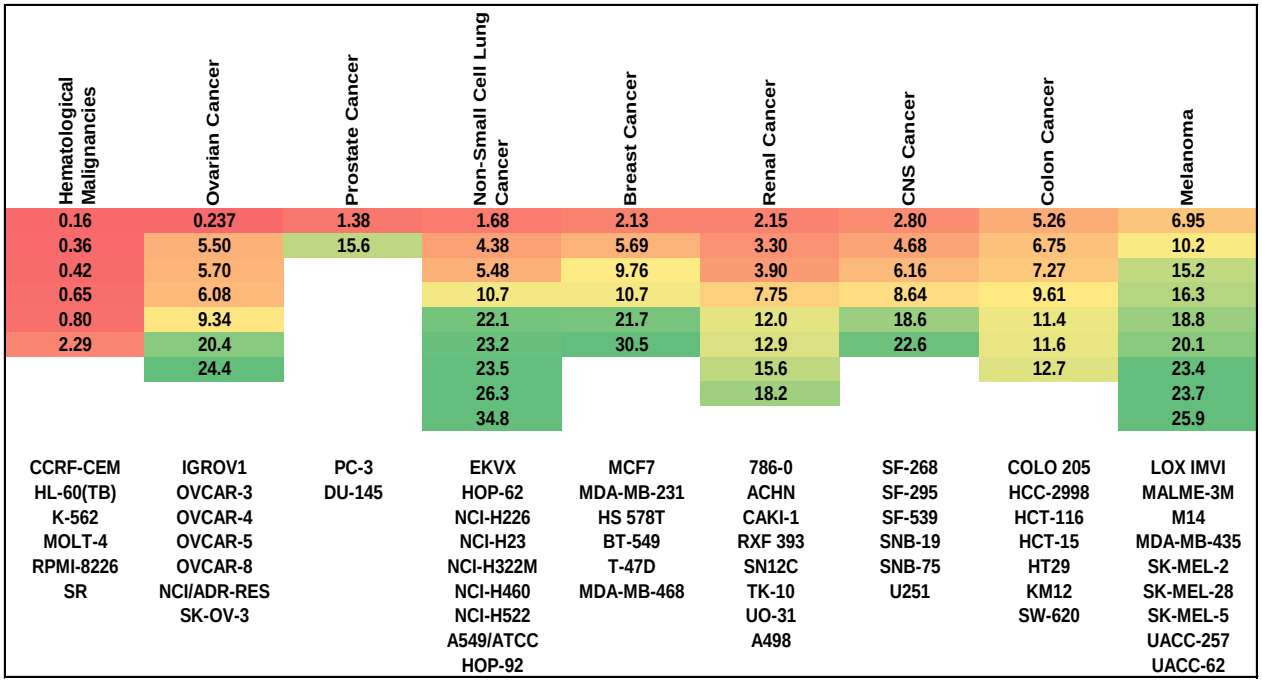

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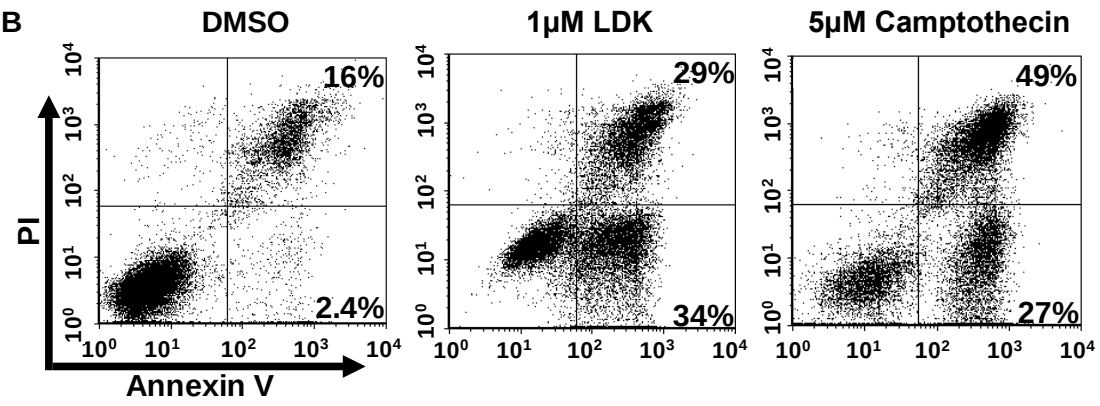

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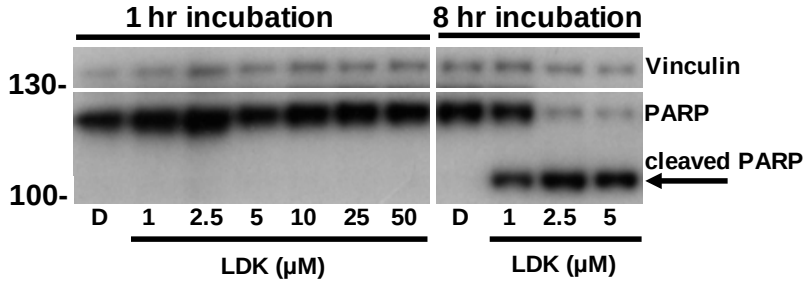

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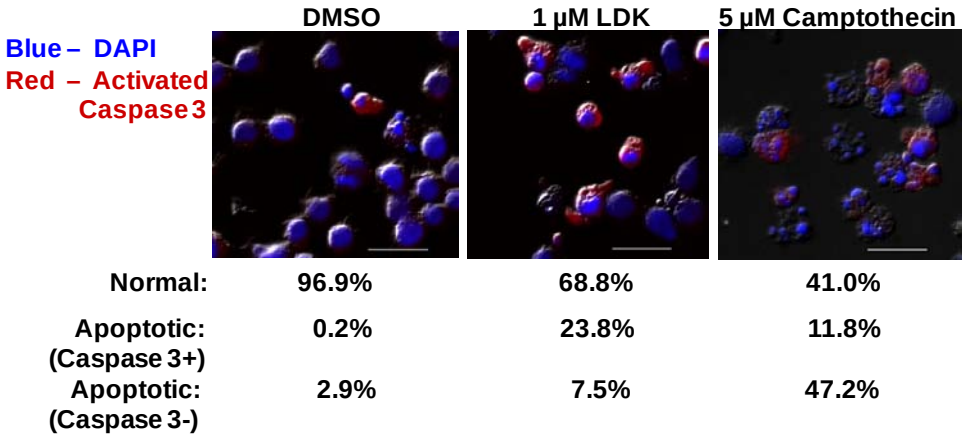
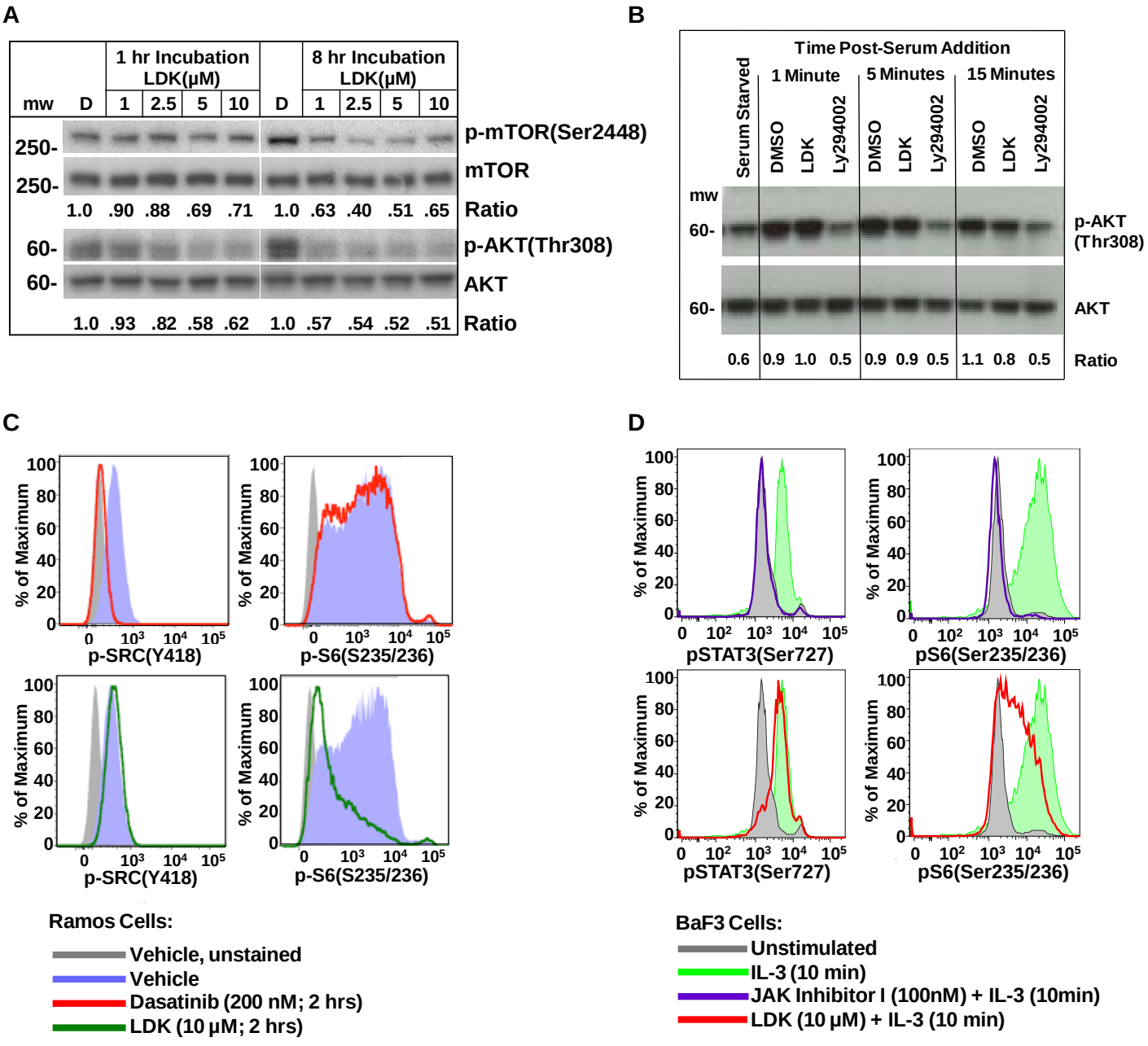
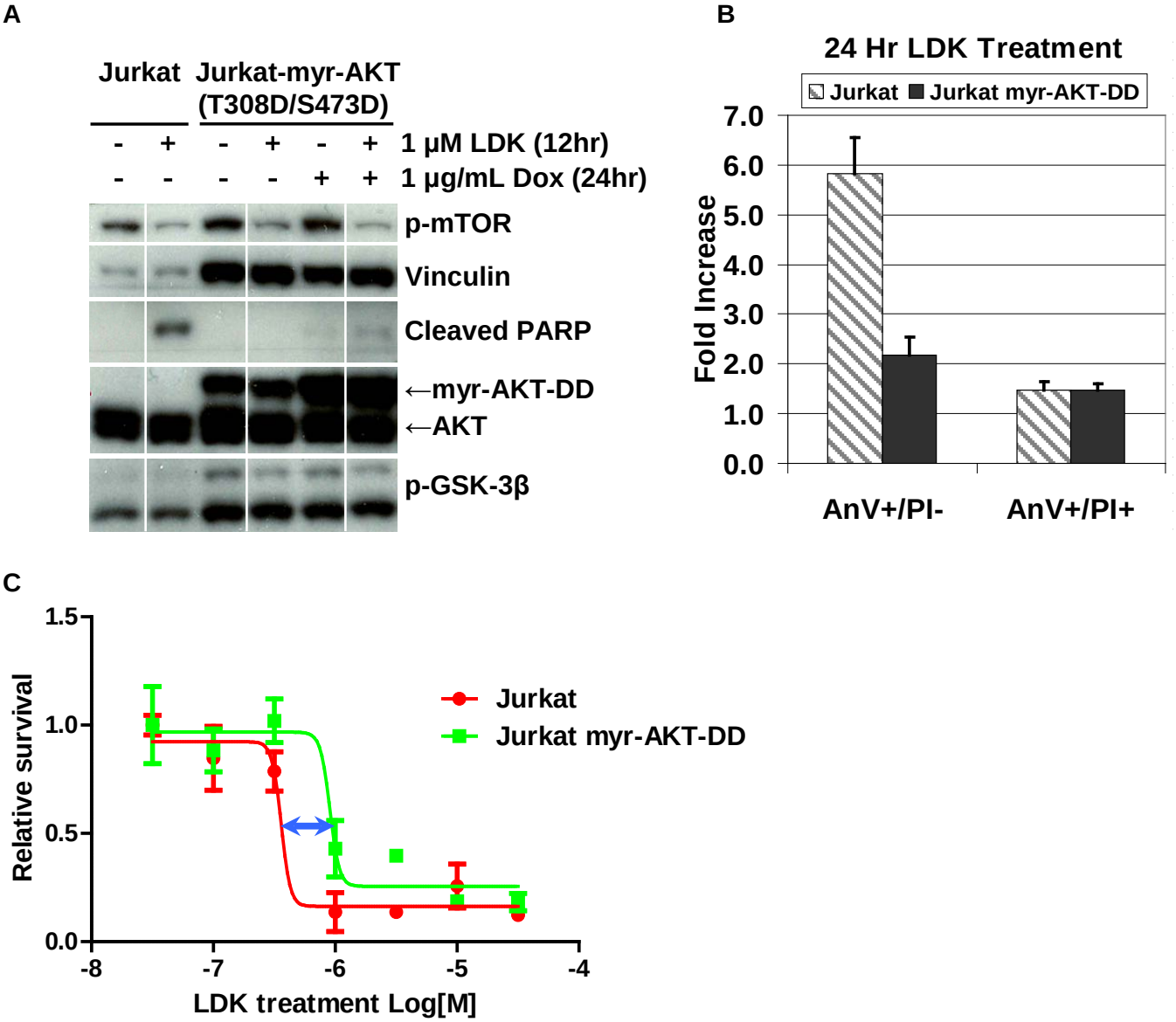


	DMSO	1 μM LDK	5 μM Camptothecin
Normal:	96.9%	68.8%	41.0%
Apoptotic: (Caspase 3+)	0.2%	23.8%	11.8%
Apoptotic: (Caspase 3-)	2.9%	7.5%	47.2%

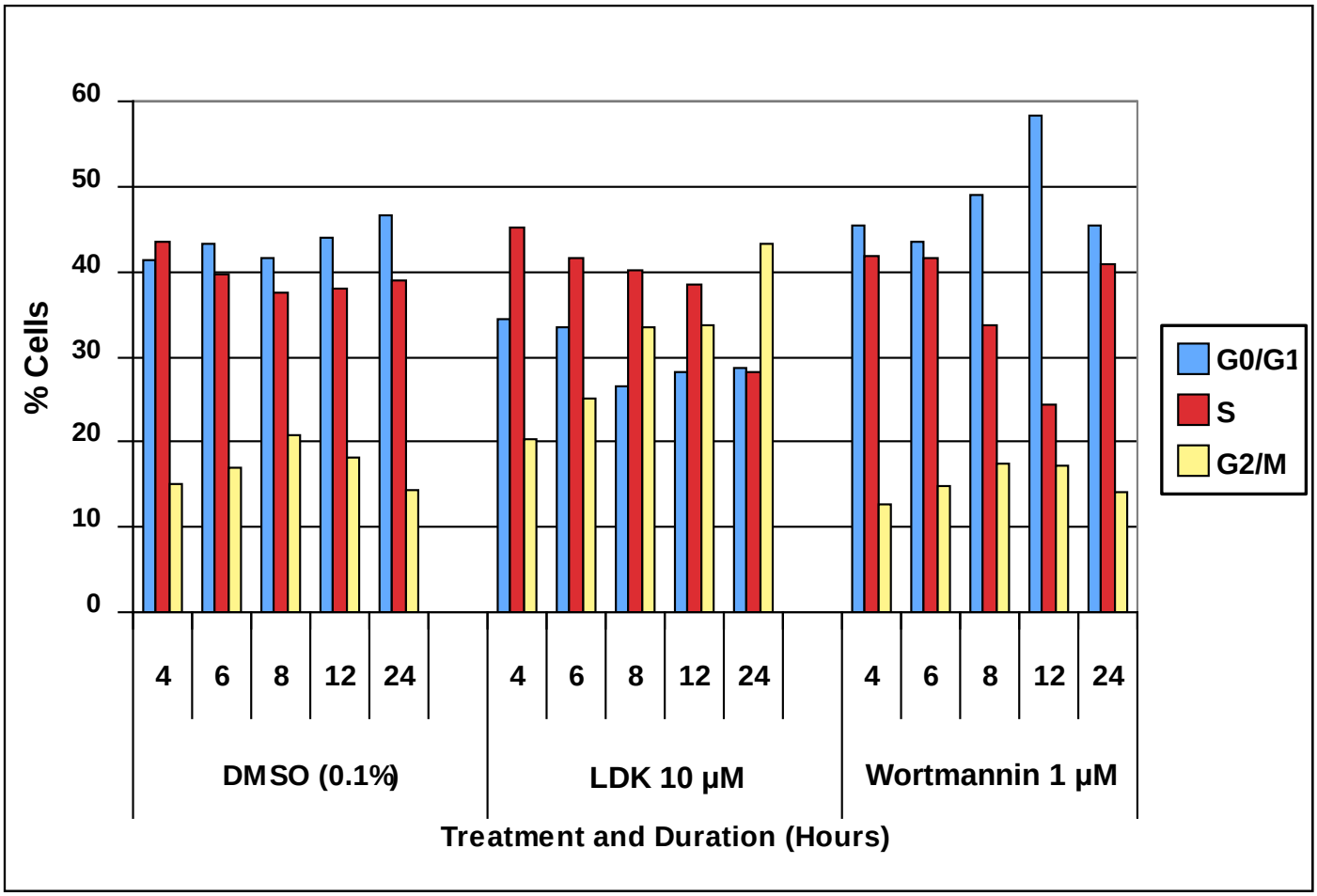
Supplemental Figure 3. **LDK-mediated reduction of AKT and mTOR phosphorylation.** (A), Dose-response of Jurkat cells to LDK treatment for one or eight hours of incubation. Phosphorylation of AKT at Thr308 and mTOR at Ser2448 was assessed by Western blot. Same blot was stripped and re-probed with antibodies for total mTOR and AKT. Densitometric ratios were normalized to 1 and 8 hours' DMSO treatment. D = DMSO. Non-contiguous sections of same gels are shown. (B) Assessment of LDK's ability to prevent serum-induced phosphorylation of serum-starved cells. CCRF-CEM T-ALL cells were serum-starved for 72 hours then pre-treated for 30 min with DMSO, LDK (10μM) or known PI3K inhibitor Ly294002 (Ly)(10μM) before addition of serum to cell media. Ly blocked any re-phosphorylation of AKT. LDK did not block serum-induced AKT re-phosphorylation and showed reduction of AKT phosphorylation first at 15 minutes post serum-stimulation. (C-D) Phosflow analysis of B-ALL line Ramos and pro-B cell line BaF3. (C) Effect of Dasatinib (top panels) and LDK (lower panels) on SRC (Y418, left panel) and S6 (S235/236, right panels) phosphorylation in B-ALL line Ramos. (D) Effects of JAK Inhibitor I (top panels) and LDK (lower panels) on STAT3 (Ser727, left panels) and S6 (S235/236, right panels) phosphorylation in pro-B cell line BaF3. For panels A and B, Western blots were scanned at room temperature using the Epson Expression 1680 scanner and software (Long Beach, CA), 16-bit grayscale acquisition, 600 dpi resolution. Image processing and densitometry was done utilizing Adobe Photoshop software v9.0.2 (San Jose, CA).



Supplemental Figure 4. LDK does not target the AKT pathway directly, but AKT inhibition is required for its toxicity in Jurkat cells. (A-C) Assessment of constitutively active myristoylated, double phosphomimetic (T308D/S473D) AKT (myr-AKT-DD) rescue of Jurkat cells from LDK treatment. (A) Western blot shows expression of myr-AKT-DD, including upregulation of AKT phosphorylation target GSK-3β, and reduction of LDK-mediated apoptosis, as indicated by reduction in cleaved PARP. (Dox = doxycycline). (B) Annexin-V/PI assessment indicates a reduction of apoptosis in LDK-treated (1μM) Jurkat (myr-AKT-DD) cells compared to Jurkat cells, relative to vehicle-treated cells. (n=3, error bars = s.e.m.) (C) Constitutively active AKT increases LDK's IC_{50} in Jurkat cells three-fold (double arrow) as determined by 24hr MTT viability assay. For panel A, Western blot was scanned at room temperature using the Epson Expression 1680 scanner and software (Long Beach, CA), 16-bit grayscale acquisition, 600 dpi resolution. Image processing and densitometry was done utilizing Adobe Photoshop software v9.0.2 (San Jose, CA).



Supplemental Figure 5. Time-course of LDK treatment shows progressive accumulation of cells in G2/M. Jurkat T-ALL cells were treated with DMSO vehicle, LDK (10μM), or Wortmannin (1μM) and cell cycle percentages were assessed over a 4-24 hour time period via flow cytometry. Wortmannin, a known inhibitor of the PI3K/AKT/mTOR pathway, shows accumulation in G0/G1 while LDK causes a progressive accumulation in G2/M.



Supplemental Figure 6. LDK treatment results in de-phosphorylation of AKT and G2/M delay in the T-ALL cell line CCRF-CEM. (A) CCRF-CEM cells were incubated for 12 hours with no treatment, DMSO (0.1%), LDK (10μM), Nocodazole (3μM), or Wortmannin (1μM) then probed via Western blot for AKT phosphorylation. Total AKT and Vinculin = loading controls, cropped non-contiguous sections of same gel. (B) CCRF-CEM cells were treated for 12 hours with LDK (10μM), Wortmannin (1μM), or Nocodazole (3μM) then stained with PI and assessed for cell cycle status via flow cytometry. For panel A, Western blots were scanned using Epson Expression 1680 scanner and software, 16-bit grayscale acquisition, 600 dpi resolution. Image processing and densitometry was done utilizing Adobe Photoshop software v9.0.2. For panel A, Western blot was scanned at room temperature using the Epson Expression 1680 scanner and software (Long Beach, CA), 16-bit grayscale acquisition, 600 dpi resolution. Image processing and densitometry was done utilizing Adobe Photoshop software v9.0.2 (San Jose, CA).

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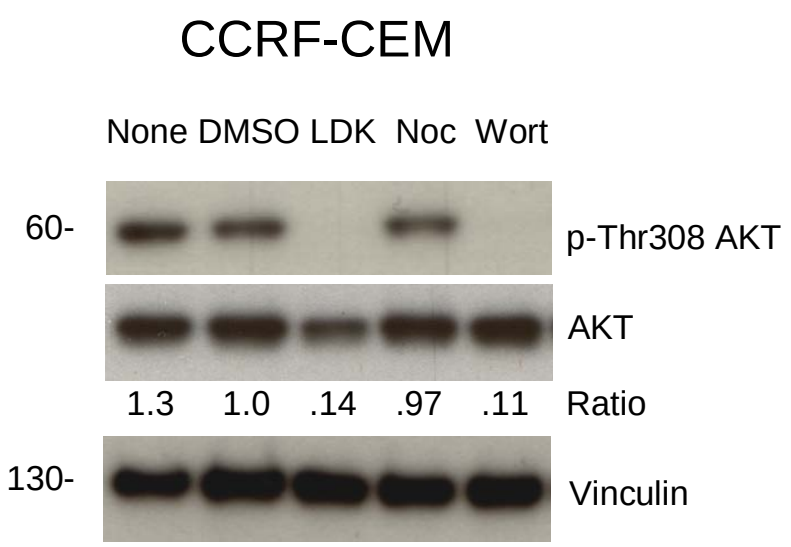

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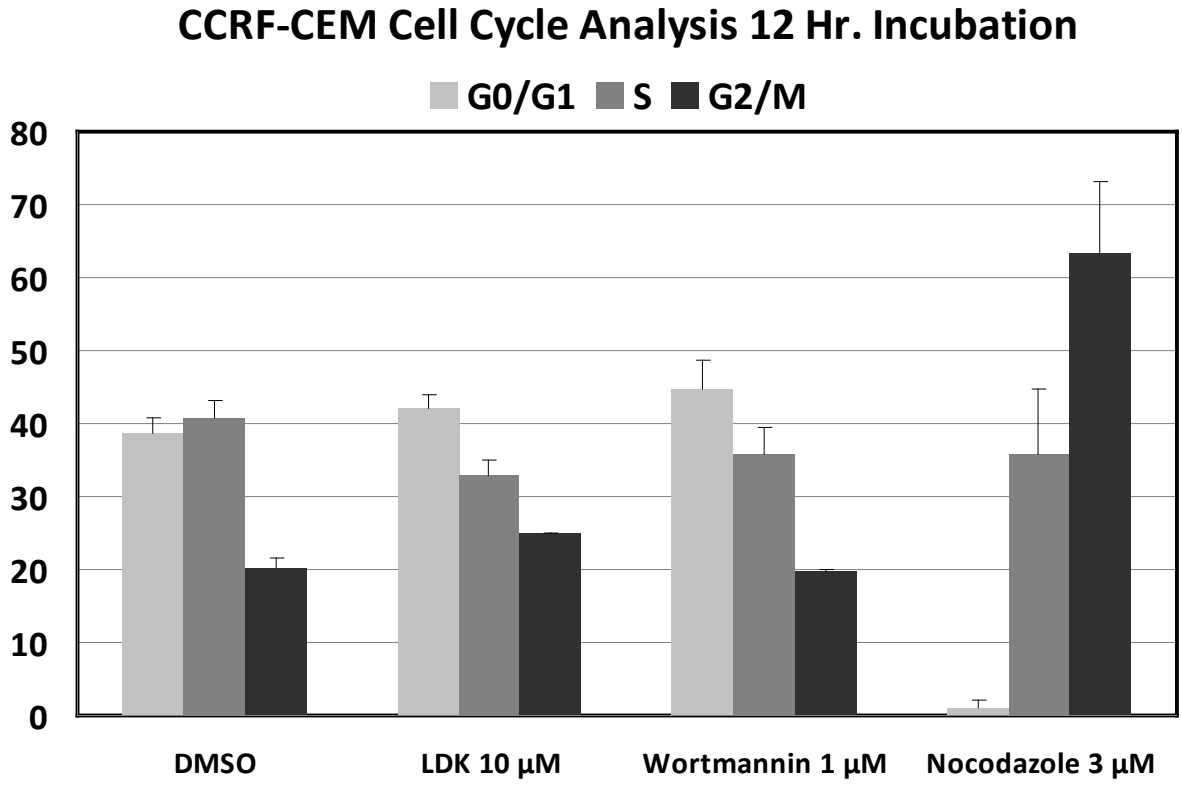

Supplemental Figure 7. Pharmacokinetics and lack of toxicity of LDK in mice. (A) LC/MS standard calibration curve for LDK detection. (B) Four groups of five male NOD-SCID mice each were injected IP b.i.d for two weeks with either vehicle or LDK at 16, 80, or 200mg/kg. After the two-week course of treatment, LDK serum levels were determined 24 hours following last treatment by liquid chromatography followed by mass spectroscopy (LC/MS). One representative serum sample per treatment level is shown. (C) Plasma concentration-time profile (mean ± s.d,; n=3) of LDK in male Swiss Albino mice following single intravenous (IV) (3mg/kg) or oral (PO) (15mg/kg) administration was determined by mass spectroscopy. Single-dose half-life of LDK was 1.7h for IV and 2h for oral administration. (D) LDK shows lack of endorgan toxicity in treated mice. Kidney and liver H&E sections were taken after 2-week treatment course from vehicle- and LDK-treated mice (45mg/kg, oral, b.i.d.) and evaluated for endorgan toxicity. Representative histological sections are shown. Scale bars = 100µm. For panel D, hematoxylin - eosin stained tissue sections were reviewed with an Olympus 4E/19753 microscope (Center Valley, PA) using lens of S Plan 10. The image was acquired using digital camera Olympus DP71 (Center Valley, PA) and was processed with DP controller.

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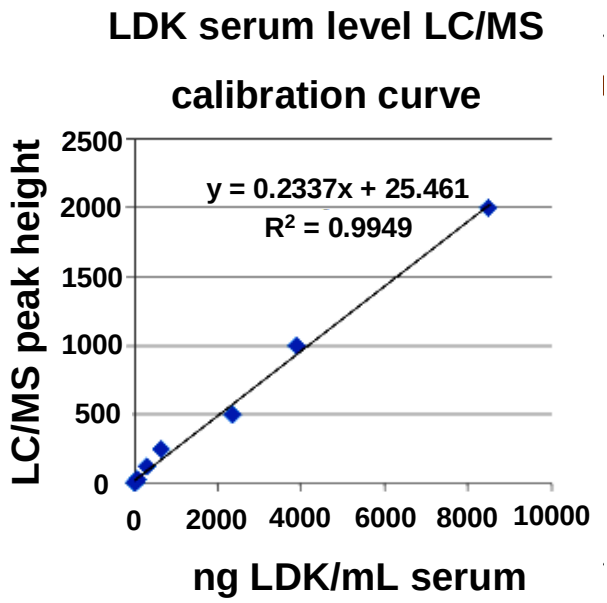

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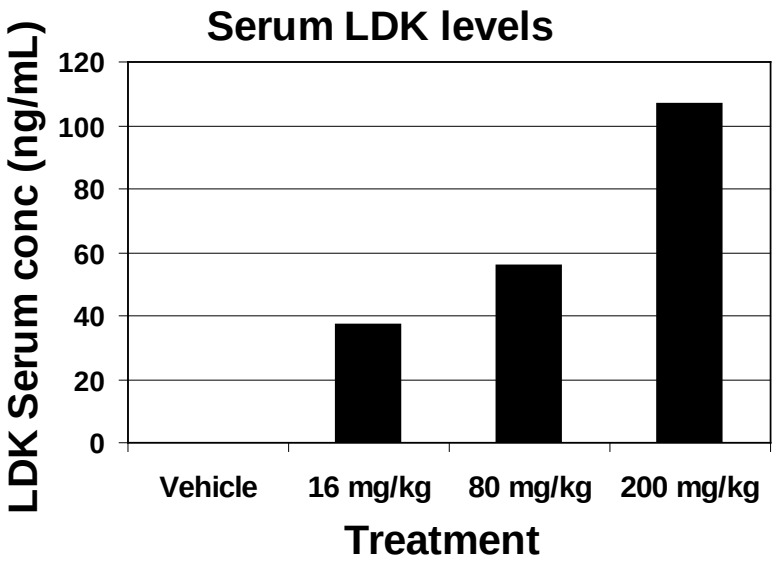

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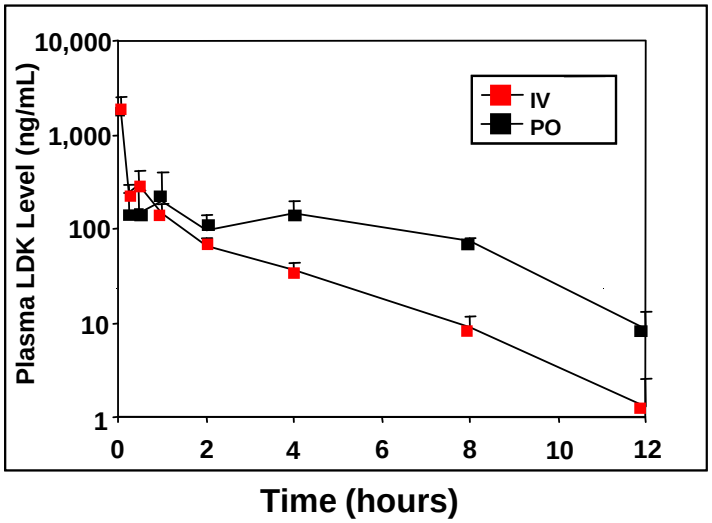

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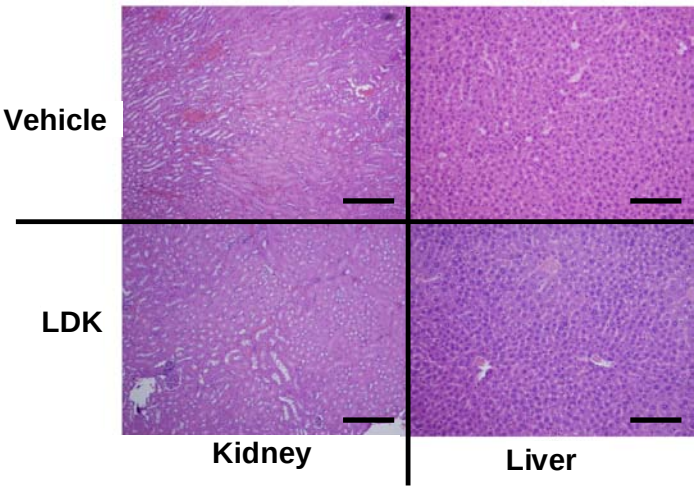
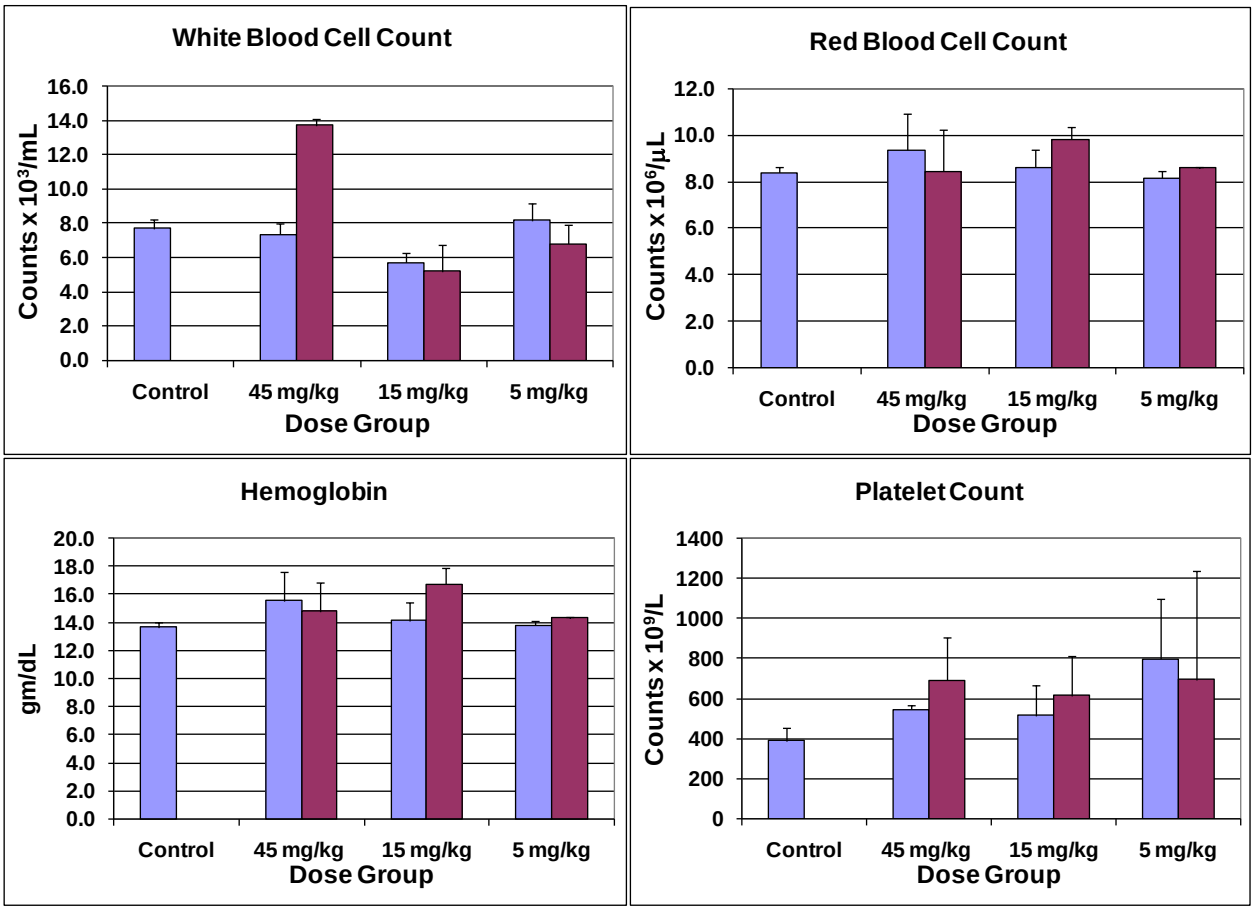


Supplemental Figure 8. LDK treatment shows no significant toxicity in complete blood count, thymus, or spleen cell counts. LDK-treated individuals (indicated doses were given orally b.i.d.) showed no significant difference when compared to diluent-only treated mice (tested on day 2) in (A) peripheral blood counts, (B) thymus and (C) spleen differential cell counts. There was a 10% drop in the percentage of CD8 single positive T lymphocytes in the thymus. Total numbers of thymocytes and overall size of thymi were reduced approximately 50%. Each data point represents average of three mice sacrificed on the indicated time point and dose. Blue bars = treatment day 2, Red bars = treatment day 14, Error bars = SD.

A



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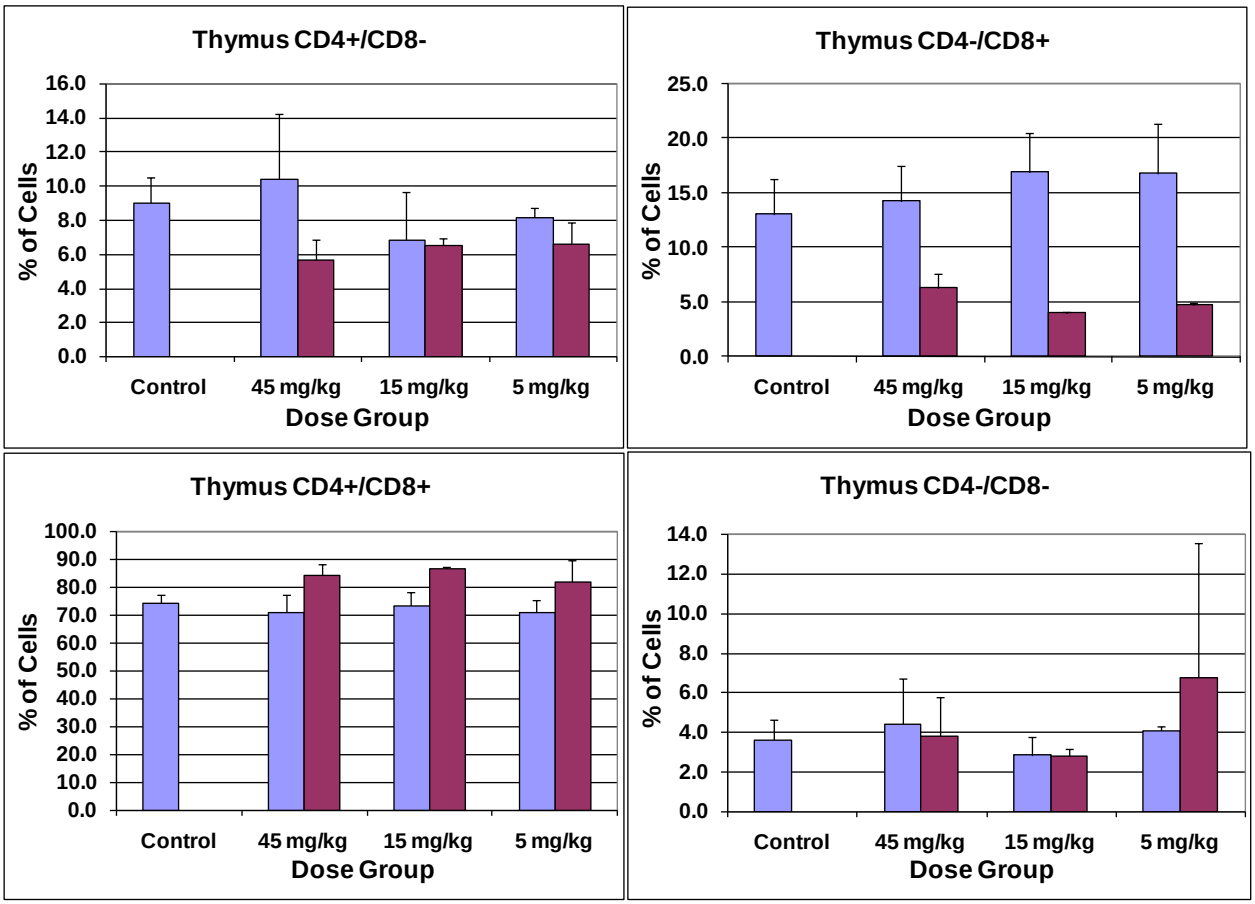
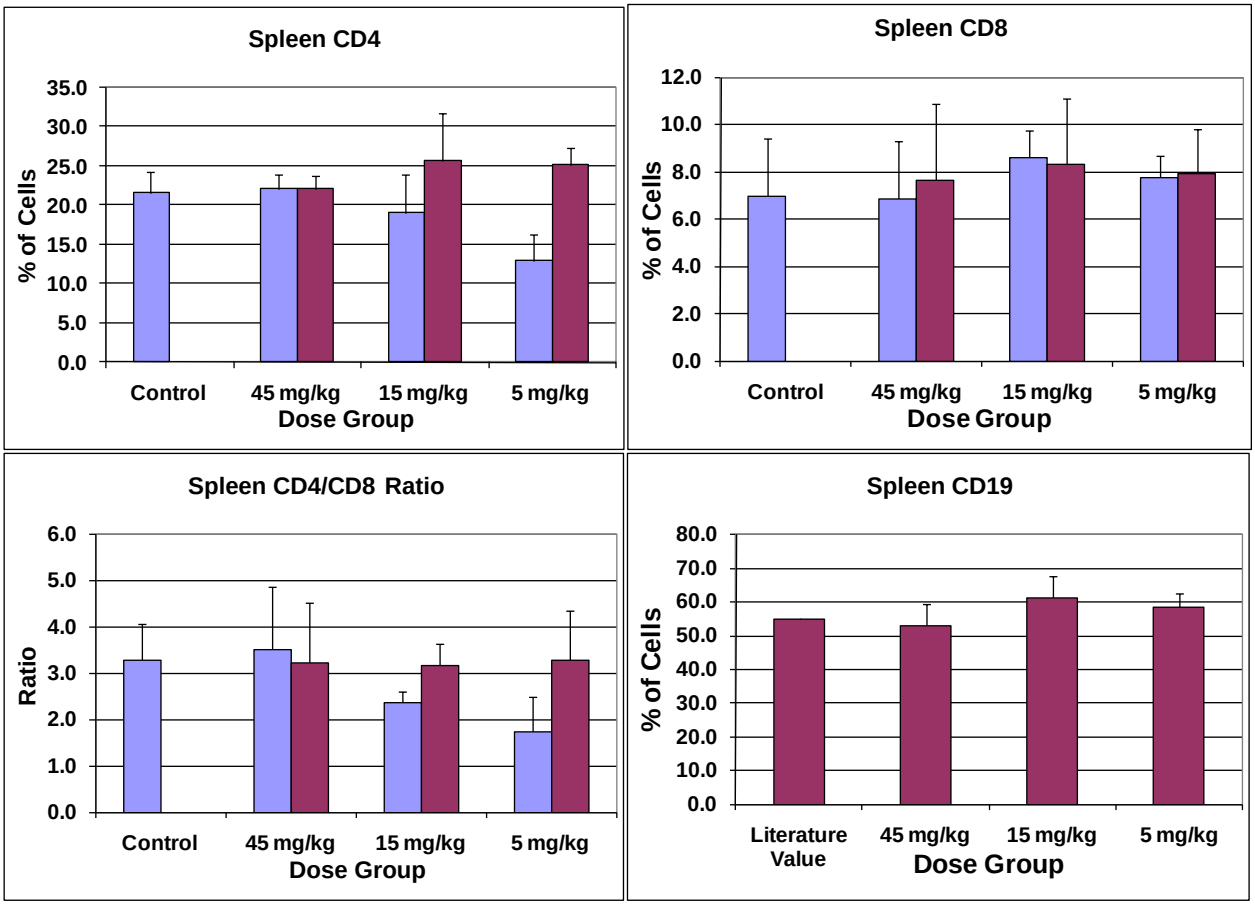
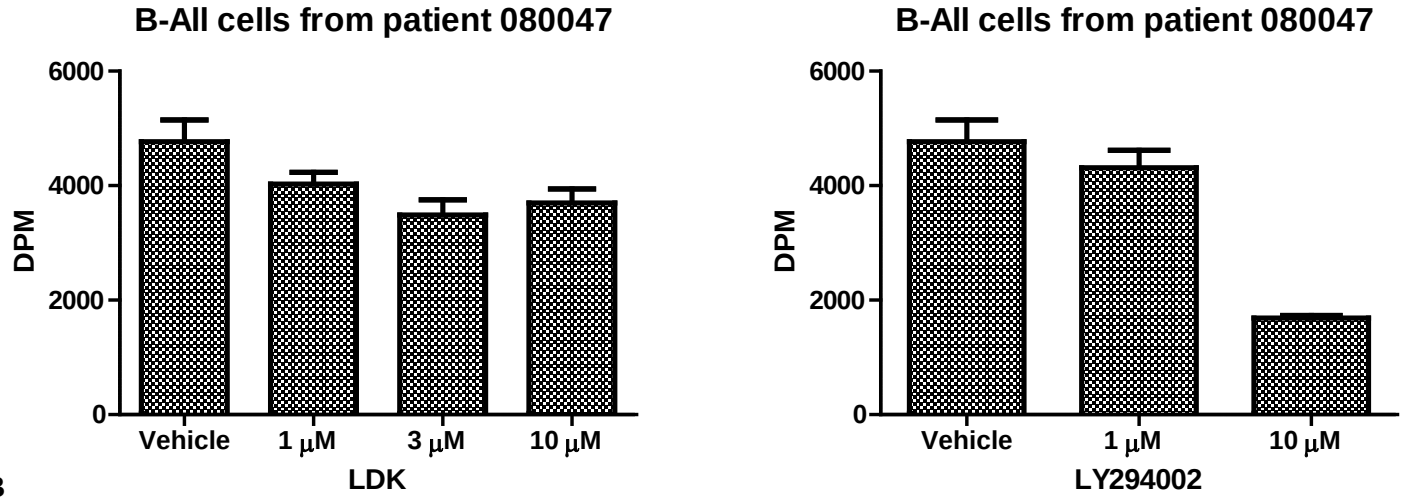

Thymus CD4+/CD8-
% of Cells
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Control
45 mg/kg
15 mg/kg
5 mg/kg
Dose Group
Thymus CD4-/CD8+
% of Cells
25.0
20.0
15.0
10.0
5.0
0.0
Control
45 mg/kg
15 mg/kg
5 mg/kg
Dose Group
Thymus CD4+/CD8+
% of Cells
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Control
45 mg/kg
15 mg/kg
5 mg/kg
Dose Group
Thymus CD4-/CD8-
% of Cells
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Control
45 mg/kg
15 mg/kg
5 mg/kg
Dose Group

C


Spleen CD4
% of Cells
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Control
45 mg/kg
15 mg/kg
5 mg/kg
Dose Group
Spleen CD8
% of Cells
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Control
45 mg/kg
15 mg/kg
5 mg/kg
Dose Group
Spleen CD4/CD8 Ratio
Ratio
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Control
45 mg/kg
15 mg/kg
5 mg/kg
Dose Group
Spleen CD19
% of Cells
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Literature Value
45 mg/kg
15 mg/kg
5 mg/kg
Dose Group

Supplemental Figure 9. Response of a collection of primary patient B-ALL samples to LDK treatment. (A-J) Ten primary human B-ALL cell samples were treated with LDK and the PI3K inhibitor LY294002 then assessed for viability via DPM measurement. Left panels – dose response of primary B-ALL sample viability after 48 hrs incubation in LDK. Right panels – dose response of primary B-ALL patient samples to treatment with LY294002. (n values as noted, error bars = s.e.m.) Full descriptions of patient samples may be found in Supplemental Table 6.

A



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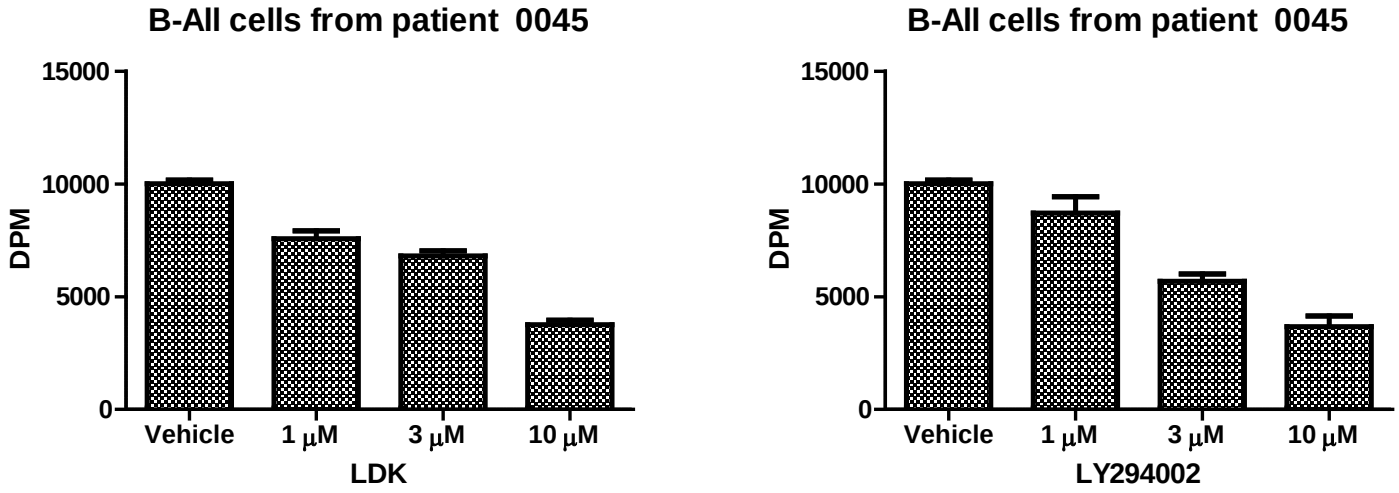

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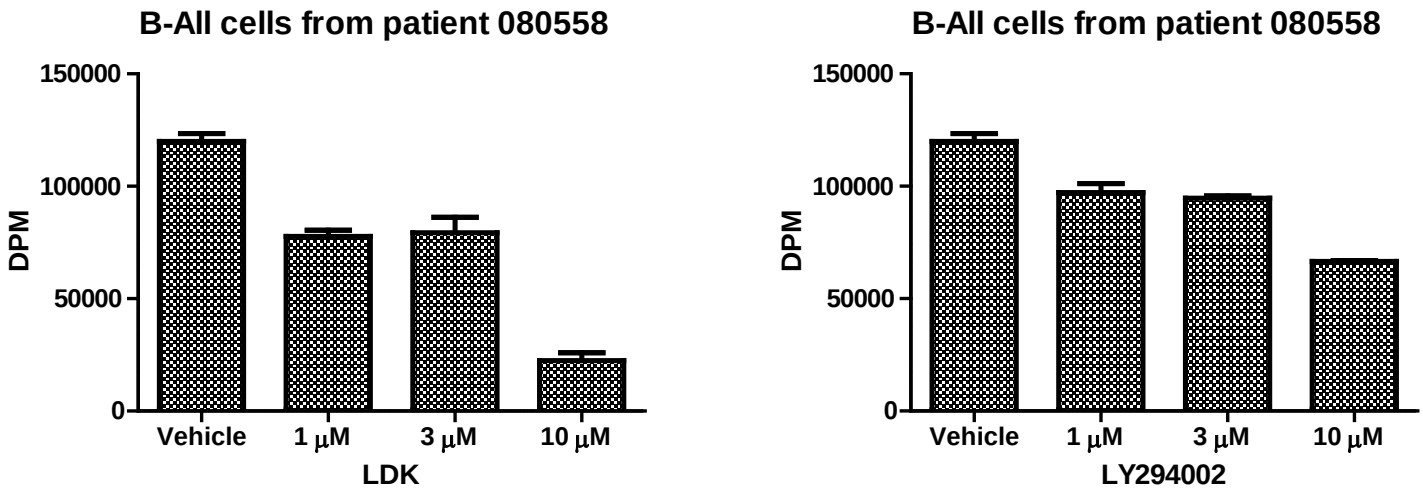
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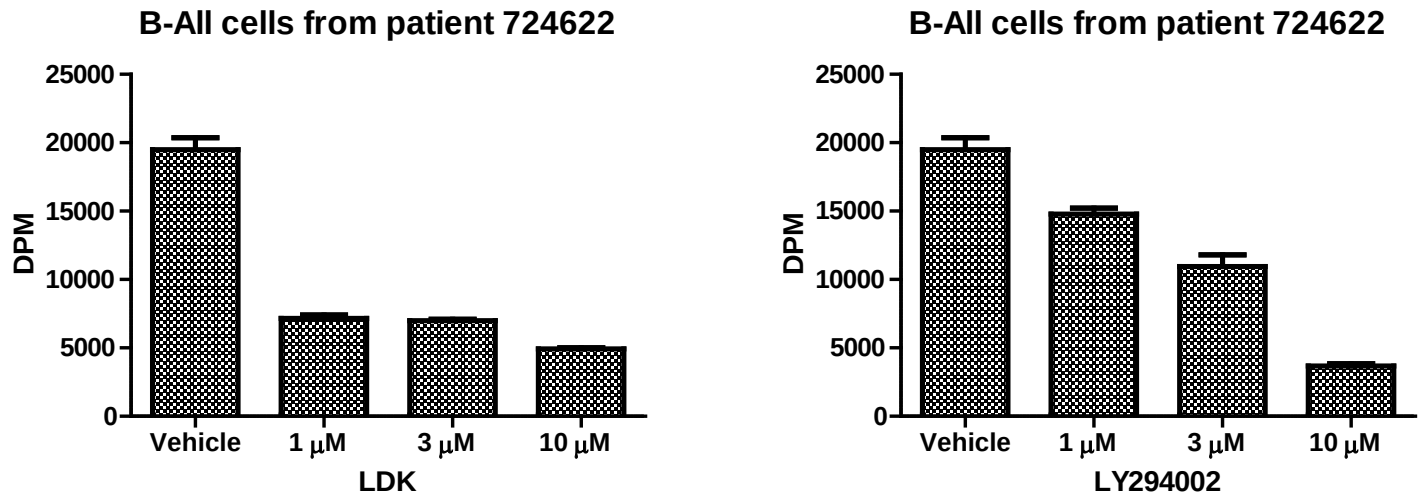
150000
100000
50000
0
DPM
Vehicle
1 μM
3 μM
10 μM
LDK
B-All cells from patient 080558
150000
100000
50000
0
DPM
Vehicle
1 μM
3 μM
10 μM
LY294002

D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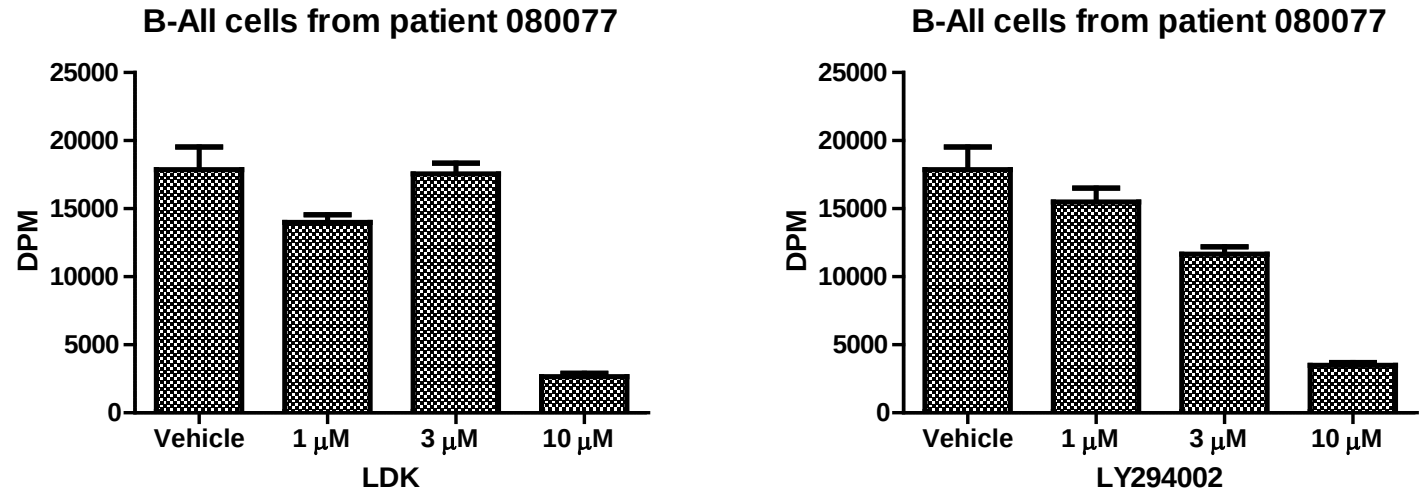
25000
20000
15000
10000
5000
0
DPM
Vehicle
1 μM
3 μM
10 μM
LDK
B-All cells from patient 724622
25000
20000
15000
10000
5000
0
DPM
Vehicle
1 μM
3 μM
10 μM
LY294002

E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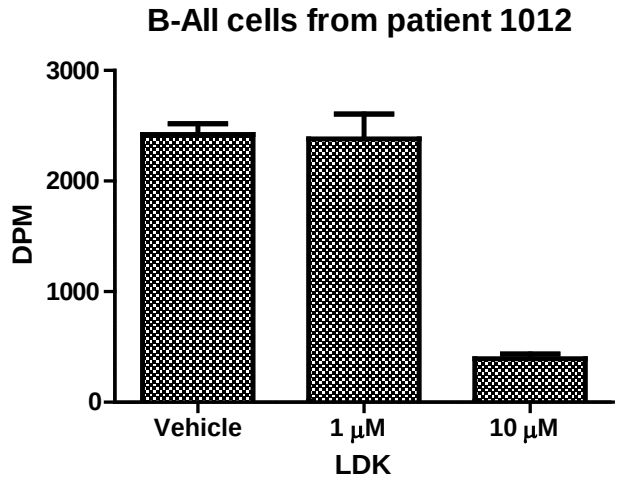
25000
20000
15000
10000
5000
0
DPM
Vehicle
1 μM
3 μM
10 μM
LDK
B-All cells from patient 080077
25000
20000
15000
10000
5000
0
DPM
Vehicle
1 μM
3 μM
10 μM
LY294002

F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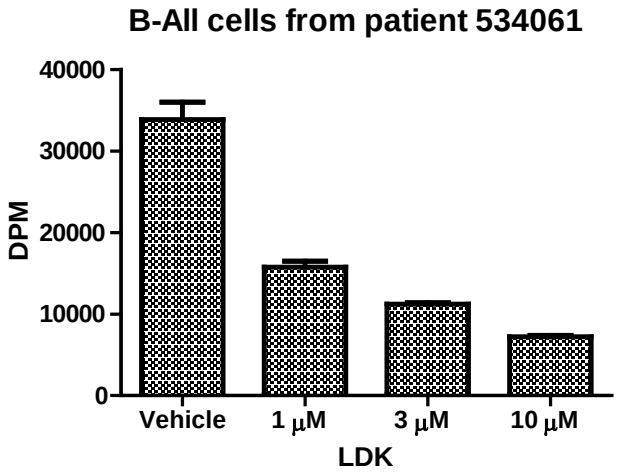
DPM
3000
2000
1000
0
Vehicle
1 μM
10 μM
LDK

G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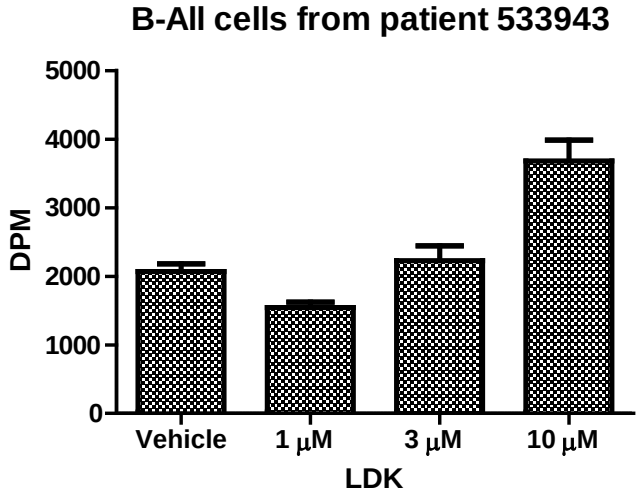
DPM
40000
30000
20000
10000
0
Vehicle
1 μM
3 μM
10 μM
LDK

H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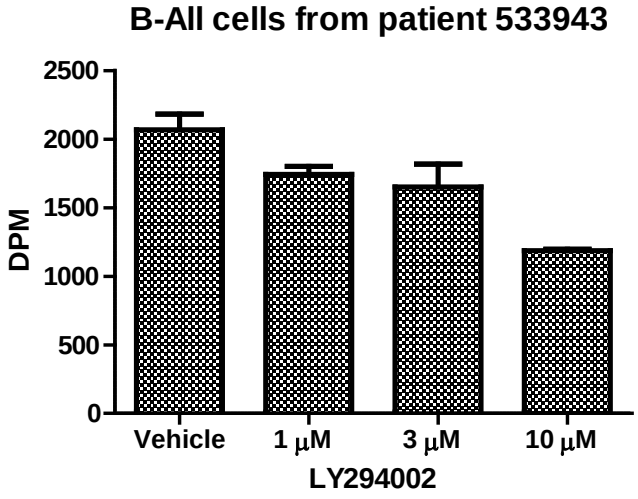
DPM
5000
4000
3000
2000
1000
0
Vehicle
1 μM
3 μM
10 μM
LDK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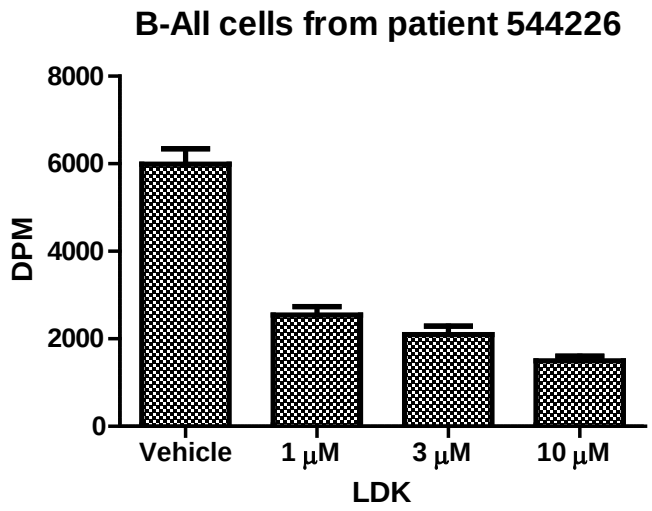
DPM
2500
2000
1500
1000
500
0
Vehicle
1 μM
3 μM
10 μM
LY294002

I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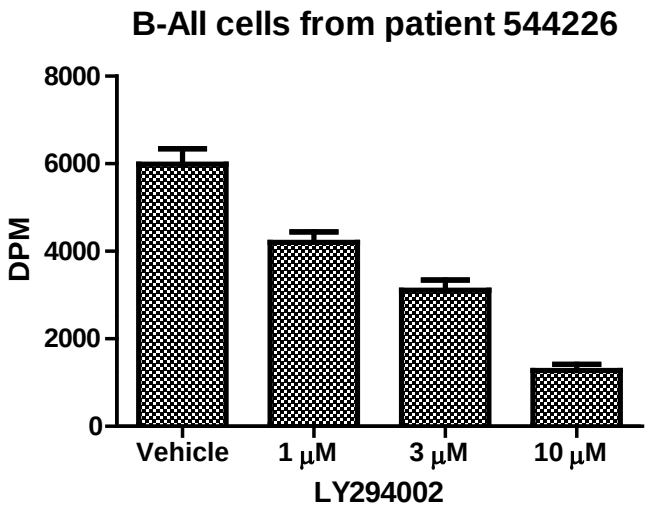
6000
4000
2000
0
DPM
Vehicle
1 μM
3 μM
10 μM
LDK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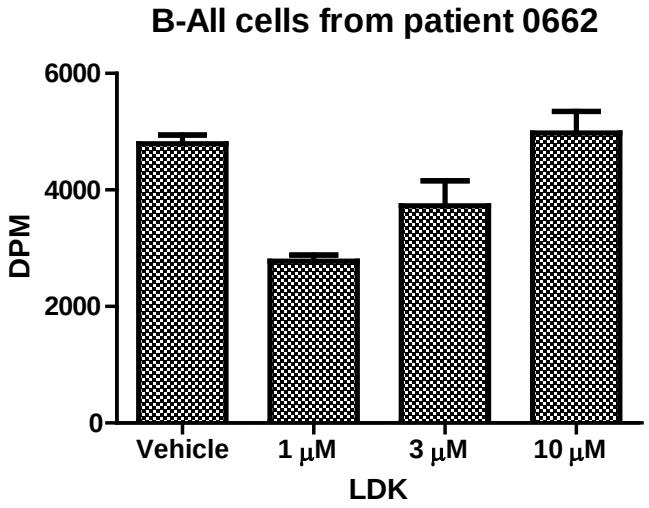
6000
4000
2000
0
DPM
Vehicle
1 μM
3 μM
10 μM
LY294002

J


B-All cells from patient 0662
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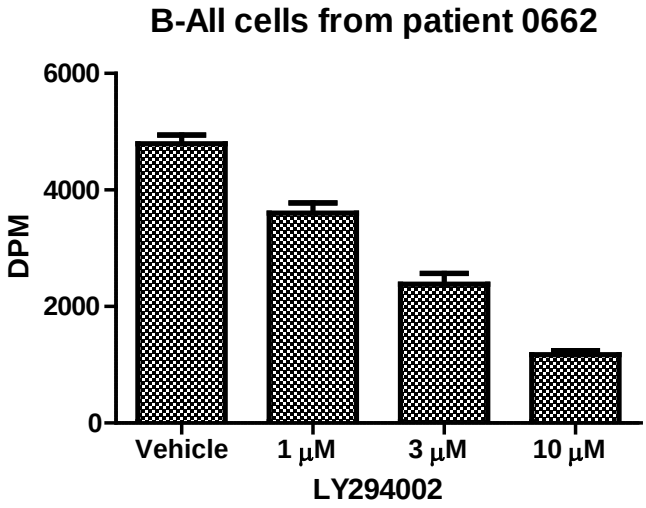
4000
2000
0
DPM
Vehicle
1 μM
3 μM
10 μM
LDK


B-All cells from patient 0662
6000
4000
2000
0
DPM
Vehicle
1 μM
3 μM
10 μM
LY294002

Supplemental Table 1. Cell cycle effects of 21 candidate compounds on zebrafish embryos. The 21 compounds with strong effects on survival of immature zebrafish T cells were assessed for their impact on the cell cycle of non-lymphoid zebrafish cells between 4 and 24 hpf. (N = No, Y = Yes, None = No effect, S = S-phase, G0/G1 = G0/G1 phase).

Strong Effect Compound Number:	Sub G1 Peak?	Delay:
1	Y	S
2	N	None
3 (LDK)	N	None
4	Y	S
5	Y	S
6	N	None
7	N	None
8	Y	S
9	N	None
10	N	G0/G1
11	N	G0/G1
12	N	G0/G1
13	N	G0/G1
14	N	G0/G1 & S
15	Y	G0/G1
16	N	G0/G1
17	N	S
18	N	G0/G1
19	Y	G0/G1
20	Y	G0/G1
21	N	G0/G1

Supplemental Table 2. LDK has minimal impact on zebrafish embryonic and larval development.
Larvae were inspected after 48 hrs treatment by light microscopy and health and development was assessed. Toxicity was ranked: Grade I – no effect, Grade II – mild edema, Grade III – sick, impaired behavior, Grade IV – lethal. (hpf = hours post-fertilization).

Age at beginning of treatment	Toxicity Ranking after 48 hrs of treatment		
	Untreated	**Vehicle**	**LDK 10μM**
24 hpf	Grade I- 96% Grade II- None Grade III- None Grade IV- 4%	Grade I- 66% Grade II- None Grade III- None Grade IV- 34%	Grade I- 35% Grade II- 35% Grade III- None Grade IV- 30%
48 hpf	Grade I- 96% Grade II- None Grade III- None Grade IV- 4%	Grade I- 88% Grade II- None Grade III- None Grade IV-12%	Grade I- ~74% Grade II- ~10% Grade III- None Grade IV- 16%
72 hpf	Grade I- 97% Grade II- None Grade III- None Grade IV- 3%	Grade I- 93% Grade II- None Grade III- None Grade IV- 7%	Grade I- ~80% Grade II- ~10% Grade III- None Grade IV- 10%
96 hpf	Grade I- 97% Grade II- None Grade III- None Grade IV- 3%	Grade I- 90% Grade II- None Grade III- None Grade IV- 10%	Grade I- ~73% Grade II- ~10% Grade III- None Grade IV- 17%
120 hpf	Grade I- 90% Grade II- None Grade III- None Grade IV- 10%	Grade I- 84% Grade II- None Grade III- None Grade IV- 16%	Grade I- 78% Grade II- None Grade III- 8% Grade IV- 14%

Supplemental Table 3. LDK is not a general kinase inhibitor. 33 kinases/phosphatases were selected for assessment of LDK activity *in vitro.* Four increasing concentrations of LDK were used per kinase or phosphatase and change in activity level was measured. The non-selective kinase inhibitor staurosporine was used as a positive control. Experiments conducted by SignalChem (Richmond, BC).

Target	%Activity change (0.2 μM LDK)	%Activity change (1 μM LDK)	%Activity change (5 μM LDK)	%Activity change (25 μM LDK)	%Activity change (1 μM Staurosporine)
AKT1	-6	-2	-9	-20	-93
AKT2	0	-7	-14	-22	-93
AKT3	0	6	-1	-1	-85
AMPK(A1/B1/G1)	-3	2	1	-1	-93
PTPRC (CD45)	1	2	-3	-6	1
c-KIT	0	1	-3	-7	-95
CAMK1	2	4	3	-1	-89
CSK	-1	-5	-6	-13	-53
ERK1	1	-1	-3	-1	-32
FAK	2	3	5	4	-97
FYN A	-3	-1	-4	-7	-92
GSK-3β	-6	-8	-15	-17	-91
HCK	-2	-6	-10	-14	-99
ITK	-2	3	2	1	-100
JNK1	-4	-3	0	2	-40
JNK2	0	-2	-6	0	-32
LCK	3	3	-2	-4	-76
LYN A	0	0	-5	-10	-96
MEK1	0	0	-11	-16	-72
p38α	3	1	1	-7	-6
p38δ	1	1	0	0	-9
PI3K (p110α/p85α)	-1	-3	-8	-9	-16
PI3K (p110β/p85α)	6	0	-4	-5	-26
PDK1	-3	-5	-2	-4	-86
PIM1	-5	-9	-18	-25	-100
PKCγ	-1	-3	-6	-8	-93
PKCθ	-3	-7	-3	-5	-99
PKCζ	1	1	1	-8	-68
SRC	-1	-11	-15	-18	-87
SYK	-1	-1	-6	-11	-95
TEC	-2	-8	-7	-11	-92
YES1	-4	-4	-7	-28	-98
ZAP70	1	2	-2	-5	-99

Supplemental Table 4. LDK treatment results in G2/M arrest for sensitive T-ALL and B-ALL cell lines. T-ALL (Jurkat, CCRF-CEM) and B-ALL (697, Nalm-6, and RS4;11) cell lines were treated with either DMSO vehicle only, LDK, or Wortmannin. All LDK-sensitive cell lines (Jurkat, CCRF-CEM, 697, Nalm-6) show G2/M accumulation upon treatment with LDK, whereas the LDK-resistant B-ALL cell line RS4;11 shows accumulation in G0/G1 upon treatment with LDK. Wortmannin, a known inhibitor of the PI3K pathway, causes G0/G1 accumulation in all lines tested with the exception of 697. Cell cycle percentages shown are average of three separate experiments ± s.d.

Cell Line	Treatment	% Cells / Phase		
		G0 / G1	S	G2 / M
Jurkat	DMSO (0.1%)	47 ± 2	38 ± 1	15 ± 1
	LDK 10 μM	44 ± 3	28 ± 2	28 ± 2
	Wortmannin 1 μM	59 ± 2	27 ± 1	15 ± 2
CCRF-CEM	DMSO (0.1%)	39 ± 2	41 ± 2	20 ± 1
	LDK 10 μM	42 ± 2	33 ± 2	25 ± 0
	Wortmannin 1 μM	45 ± 4	36 ± 4	20 ± 0
697	DMSO (0.1%)	42 ± 3	35 ± 6	23 ± 3
	LDK 10 μM	32 ± 4	30 ± 11	39 ± 7
	Wortmannin 1 μM	42 ± 6	34 ± 7	24 ± 1
Nalm-6	DMSO (0.1%)	44 ± 2	45 ± 2	11 ± 1
	LDK 10 μM	44 ± 4	32 ± 1	24 ± 6
	Wortmannin 1 μM	56 ± 2	33 ± 2	10 ± 1
RS4;11	DMSO (0.1%)	49 ± 1	43 ± 2	8 ± 4
	LDK 10 μM	65 ± 3	27 ± 3	9 ± 1
	Wortmannin 1 μM	67 ± 5	27 ± 5	6 ± 1

Supplemental Table 5. Characteristics of primary BCR-ABL translocated B-ALL and CML patient samples and growth inhibition by LDK treatment.

Sample ID (Type)	Gender / Age	Cytogenetics	BCR-ABL1	BCR-ABL1 mutation	Cell Isolation / Clinical Course	MTT Assay LDK IC_{50}
LAX2 (Ph+ B-ALL)	m/38	t(9;22)(q34;q11)	p210	T315I	Relapse (on Imatinib)	0.814 µM
SFO2 (Ph+ B-ALL)	m/7 m/8	t(9;22)(q34;q11)	p210	unmutated	Diagnosis and Relapse (on Nilotinib)	0.762 µM
BLQ5 (Ph+ B-ALL)	f	FISH der(9), der(22)	p190	T315I	Relapse (on Imatinib)	0.939 µM
TXL3 (Ph+ B-ALL)	N/A	t(9;22)(q34;q11)	p210	unmutated	Diagnosis	0.888 µM
ICN1 (Ph+ B-ALL)	N/A	t(9;22)(q34;q11)	p210	unmutated	Diagnosis	0.795 µM

Sample ID	Leukemia Type	Age at Diagnosis	Gender	Cytogenetics	Source (BM or PB)	BCR-ABL1 mutation	Prognosis/ Outcome
10-003	Ph+ CML	43	M	BCR-ABL	PB	Unmutated	Alive
11-007	Ph+ CML	N/A	F	BCR-ABL (T315I)	PB	(T315I)	Alive
11-008	Ph+ B-ALL	62	M	Complex Karyotype	PB	Complex Karyotype	Alive
11-018	Ph+ B-ALL	N/A	M	BCR-ABL	PB	Unmutated	Alive
11-032	Ph+ B-ALL	62	M	Complex Karyotype	PB	Complex Karyotype	Alive

Supplemental Table 6. Characteristics of primary B-ALL patient samples and LDK-mediated inhibition of proliferation.

Sample ID	Age at Diagnosis	Gender	PB WBC Count (109)	Source (BM or PB)	Cytogenetics	Prognosis / Outcome
80047	55	Female	129	PB	MLL rearrangent	Alive
45	67	Female	79	PB	BCR-ABL	Deceased
80558	46	Female	22	PB	BCR-ABL (p190)	Alive
724622	46	Male	135	PB	BCR-ABL	Deceased
80077	21	Male	5	BM	t(2;3)	Deceased
534061	44	Male	N/A	N/A	N/A	Deceased
533943	25	Male	N/A	N/A	N/A	Deceased
544226	57	Female	N/A	N/A	N/A	Deceased
662	30	Male	89	PB	xy del. 17 (p11.2)	Deceased
1012	16	Male	9	BM	Normal	Alive

Sample ID	LDK			Ly294002		
	1 µM	3 µM	10 µM	1 µM	3 µM	10 µM
80047	16 ± 4	27 ± 6	23 ± 5	10 ± 6	N/A	65 ± 1
45	24 ± 4	32 ± 2	63 ± 3	13 ± 7	43 ± 3	63 ± 5
80558	35 ± 2	34 ±6	81 ± 3	19 ± 3	21 ± 1	44 ± 0
724622	63 ± 1	64 ± 1	75 ± 1	24 ± 2	44 ± 4	81 ± 1
80077	22 ± 3	2 ± 4	85 ± 2	13 ± 6	35 ± 3	81 ± 1
534061	54 ± 2	67 ± 1	79 ± 1	N/A	N/A	N/A
533943	25 ± 4	-8 ± 11	-78 ± 15	16 ± 3	20 ± 8	43 ±1
544226	58 ± 3	65 ± 3	75 ± 2	30 ± 4	48 ± 4	79 ± 2
662	42 ± 2	22 ± 9	-4 ± 8	25 ± 4	50 ± 4	76 ± 2
1012	2 ± 9	N/A	84 ± 2	N/A	N/A	N/A

Percent inhibition +/- s.e.m., normalized to vehicle control

Supplemental Methods

Cell lines and cell culture

All cells were cultured in media containing 100U/mL Penicillin (Invitrogen) and 100µg/mL Streptomycin (Invitrogen) and incubated at 37°C and 5% CO_2. T-ALL cell lines Jurkat, MOLT-3, HPB-ALL and CCRF-CEM were grown in RPMI 1640 media (Invitrogen) supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA). CCRF-CEM-Luc was grown in the presence of 0.3 mg/mL Geneticin. The T-ALL cell lines HPB-ALL and CCRF-CEM-Luc were a kind gift of Adolfo Ferrando (Columbia University, New York, NY). B-ALL cell lines 697, Nalm-6, Kasumi-2, and RS4;11 were grown in RPMI 1640 media supplemented with 20% FBS. RS4;11 was a kind gift of Andrew Kung (DFCI, Boston, MA). U138 (glioblastoma), Lox (melanoma), and SW480 (colon cancer) were grown in DMEM media supplemented with 10% FBS and 2mM L-glutamine. All cell lines were cultured in media containing 100U/mL Penicillin (Invitrogen) and 100µg/mL Streptomycin (Invitrogen) and incubated at 37°C and 5% CO_2.

In vitro drug treatment

Lenaldekar was initially obtained from the ChemBridge DIVERSet® library (ChemBridge, San Diego, CA). Subsequently LDK was manufactured by the University of Utah Chemistry Department (M.S., K.B.) using the following components: hydroxyquinoline (Sigma-Aldrich), hydrazine hydrate (Sigma-Aldrich), and indole-3-carboxaldehyde (Sigma-Aldrich). Camptothecin, Nocodazole, imatinib, γ-secretase inhibitor IX, dexamethasone, LY294002, UCN-01 and Wortmannin were obtained from Sigma-Aldrich. AKT Inhibitor IV and Doxorubicin were obtained from Calbiochem. Taxol was obtained from VWR (Radnor, PA). Drugs were dissolved in 100% DMSO then diluted for experimentation such that the final concentration of DMSO did not exceed 0.1%.

MTT assays

Cells were plated in 96-well plates at a density of 25,000 cells per well for RS4;11, 100,000 cells per well for peripheral blood T-cells, and 50,000 cells per well for all other cell types. Cells were exposed to treatment conditions for the indicated durations at 37°C and 5% CO_2 then incubated with thiazolyl blue tetrazolium bromide (MTT) reagent (Sigma-Aldrich Corp., St. Louis, MO) for an additional 3 hours. Each well was washed one time with HBSS then DMSO was added to dissolve the formazan crystals. Absorbance values were then

measured at 570nm with a Bio-Rad microplate spectrophotometer (Bio-Rad, Hercules, CA). The absorbance values were background subtracted and normalized to vehicle (DMSO) treated values. Concentration response curves were generated using non-linear regression with the GraphPad Prism® software package (GraphPad Software, Inc., La Jolla, CA) to generate IC_{50} values.

Viability of $Atm^{-/-}$ mouse primary T-ALL cell lines

A detailed characterization of these lines will be published elsewhere. Briefly, IL-7-dependent cell lines were established from primary T-ALLs arising in *$Atm^{-/-}$* mice. Expression of PTEN and activated NOTCH1 in the primary T-ALLs was evaluated by western blotting. Cells were cultured for 24 and 48 hours in IL-7 with 10μM γ-secretase inhibitor IX (Calbiochem, Gibbstown, NJ), LDK, or DMSO vehicle. Cell viability was determined using the Cell Titre Blue® Cell Viability Assay (Promega, Madison, WI) following the manufacturer's instructions.

Peripheral blood T- and B-Cell isolation

All cells were cultured in media containing 100U/mL Penicillin (Invitrogen) and 100μg/mL Streptomycin (Invitrogen) and incubated at 37°C and 5% CO_2. Blood was drawn into BD Vacutainer K2 EDTA blood collection tubes (Becton Dickinson). Blood was diluted in an equal volume of PBS, layered over lymphocyte separation medium (Cellgro, Manassas, VA), and centrifuged at 1400 rpm for 20 minutes. The mononuclear lymphocyte layer was transferred to a new tube, diluted with PBS, and centrifuged to pellet the lymphocytes. The lymphocyte pellet was diluted in 5 mL of RPMI 1640 media supplemented with 20% FBS, 100U/mL penicillin, and 100μg/mL streptomycin; transferred to a T25 cell culture flask, and incubated overnight at 37°C and 5% CO_2. The following day media was removed by centrifugation and cells were suspended at a density of 1×10^8 cells/mL. B-cells were isolated using the EasySep CD19 positive selection kit from StemCell Technologies (Vancouver, BC). Isolated B-Cells were cultured in media with 100ng/mL Interleukin-10 (Prospec, East Brunswick, NJ) for experimental procedures. The remaining cell suspension was then subject to T-Cell isolation using the EasySep CD3 positive selection kit from StemCell Technologies. Isolated T-Cells were cultured in media with 30IU/mL Interleukin-2 (Hoffman-La Roche Inc., Nutley, NJ) for subsequent experimental procedures.

***In vitro* apoptosis assay**

Jurkat cells were treated with DMSO vehicle (0.1%), 1µM LDK or 5µM Camptothecin for 24 hours then stained with propidium iodide and Annexin V-FITC (BD Pharmingen) according to the manufacturer's instructions. Apoptosis was determined via flow cytometry in a FACScan analyzer (Becton Dickinson).

Western blot analysis

Cells were treated with the indicated compounds, pelleted and washed with cold PBS. Protein was extracted via 10 min incubation on ice in cell lysis buffer (25mM TRIS pH 7.4, 150mM NaCl, 1mM $CaCl_2$, 1% Triton X-100) with phosphatase (Sigma-Aldrich) and protease (Sigma-Aldrich) inhibitors. Protein density was determined via Bio-Rad Protein assay. Proteins were separated via gel electrophoresis on SDS-PAGE gels (Invitrogen). Protein was then transferred to PVDF membrane and the membrane incubated in milk block (5% non-fat dry milk powder in 1% PBS-T) for either 1 hour at room temperature or overnight at 4°C. Membranes were then incubated for either 1-3 hours at room temperature or over night at 4°C in primary antibody diluted in primary antibody dilution buffer (5% BSA, 0.1% Tween 20 and 0.01% NaN_3 in PBS, filtered at 0.45µm). Primary antibodies purchased from Cell Signaling included PARP, AKT, p-AKT(Ser473), p-AKT(Thr308), mTOR, p-mTOR(Ser2448), PTEN, p70S6K, p-p70S6K(Thr389), Cyclin A and Cyclin B1. Phospho-Histone H3 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Actin primary antibody was purchased from ImmunO MP Biomedicals (Solon, OH). Vinculin antibody was purchased from Sigma. All primary antibodies were utilized at a 1:1,000 dilution ratio in primary antibody dilution buffer with the exceptions of vinculin (1:5,000) and p-AKT(Ser473) (1:2,000). Blots were then washed 3x for 10 min each in PBS-T and incubated in secondary antibody. Secondary antibodies used were sheep-anti-mouse (Amersham, Piscataway, NJ) and donkey-anti rabbit (Amersham) at a 1:10,000 dilution in milk block for 1 hour at room temperature. Blots were then washed again 3x for 10 min each in PBS-T. Chemiluminescence was assessed using WESTERN LIGHTNING-*Plus*-ECL horseradish peroxidase (PerkinElmer, Waltham, MA) and exposed to Amersham Hyperfilm ECL film (GE Healthcare, Piscataway, NJ). Densitometry was done utilizing Adobe Photoshop CS2 v9.0.2 histogram feature.

Immunocytochemistry

Jurkat cells were treated with test compounds or DMSO vehicle for 16 hours at 37° C. Samples were fixed in 4% paraformaldehyde for 20 minutes and then permeabilized with 0.1% Triton X-100 for 15 minutes. Samples

were then washed and resuspended in PBS/1%BSA and spun onto glass slides with a Shandon Cytospin3 cytocentrifuge (Thermo Scientific, Asheville, NC) at 600 rpm for 5 minutes. Slides were blocked in TBS buffer with 0.1% Triton X-100, 2% BSA, and 0.1% azide for 10 minutes, then incubated with active caspase-3 rabbit polyclonal antibody (BD Bioscience, San Jose, CA) diluted 1:200 in PBS/1%BSA for 60 minutes at room temperature. Slides were washed 3 times, then incubated with Alexa Fluor 568 goat anti-rabbit IgG secondary antibody (Invitrogen) diluted 1:1000 in PBS/1%BSA for 60 minutes at room temperature. Slides were washed 3 times then incubated with 50nM DAPI (Sigma) for 3 minutes. Slides were washed, allowed to dry briefly, and a coverslip was mounted to each slide with a drop of Prolong Gold (Invitrogen) and allowed to dry overnight. Slides were then imaged on a Nikon eclipse E600 fluorescence microscope (Nikon Instruments Inc., Melville, NY).

Statistical analyses

For mouse xenograft results, p-values were calculated utilizing Wilcoxon rank sum test (non-parametric test) due to non-normal distribution of tumor emittence data. Alpha level = 0.05, two-tailed test. For each p-value, comparison of interest was vehicle control vs. LDK-treated emittance for the pertinent experimental timepoint.

Phosflow experiments

Phosflow staining of Ramos B cell lymphoma and IL-3-dependent BaF3 pro-B cell lines was performed using BD Phosflow antibodies (BD BioSciences, San Diego, CA), BD Fix Buffer I and BD Perm Buffer III according to the manufacturer's protocol. Non-viable cells were excluded based on staining with Fixable Blue (Invitrogen, Carlsbad, CA) used according to the manufacturer's protocol. Dasatinib was purchased from Toronto Research Chemicals (Toronto, ON Canada) and JAK Inhibitor I was purchased from Calbiochem. Immunofluorescence was analyzed using a SORP LSRII (BD Biosciences) equipped with 4 lasers emitting at 488nm (100mW), 633nm (20mW), 405nm (25mW) and 355nM (25mW).

AKT double phosphomimetic mutagenesis.

The pTRE2-hyg vector (Clontech) containing myristoylated AKT was a kind gift of Steven Grant, MD (Virginia Commonwealth University) and has been described previously (Supplemental Reference 1). AKT double phosphomimetic mutations (T308D/S473D) were introduced into the myr-AKT construct using Agilent

Technologies QuikChange Lightning™ Multi-Site Diected Mutagenesis Kit (Cat# 210515-5) according to the manufacturer's instructions. For AKT(T308D) mutagenesis, the primer used was 5'-GGTGCCACTATGAAG**GA**CTTCTGCGGAAC GCCG-3'. For AKT(S473D) mutagenesis, the primer used was 5'-ACTTCCCCCAGTTC**GA**CTACTCA GCCAGTGG-3' (mutated bases shown in bold font). Sequence-verified myristoylated AKT(T308D/S473D) constructs (myr-AKT-DD) were introduced into Jurkat cells via nucleofection using the LONZA Amaxa™ Cell Line Nucleofector Kit V (Cat# VCA-1003) according to the manufacturer's instructions for optimized delivery to Jurkat Clone E6.1 cells. Nucleofected cells were then selected in 0.5mg/mL hygromycin for two weeks. The pLVX-Tet-On (Clontech) tetracycline-response control vector was utilized to generate lentivirus via standard methods in HEK293 cells. Jurkat cells previously selected in hygromycin for stable expression of the myr-AKT-DD construct were transduced with pLVX-Tet-On carrying lentivirus then selected in dual 0.5mg/mL neomycin/hygromycin for two weeks before testing for myr-AKT-DD transgene expression and LDK resistance.

***In vitro* kinase assay**

In vitro kinase assay was performed by SignalChem, Inc. (Richmond, BC). LDK was tested in triplicate against 33 purified kinase/phosphatases (See Supplemental Table 3) and their peptide targets, utilizing four increasing LDK concentrations of 0.2µM, 1µM, 5µM, and 25µM. Read-out for kinase assay was incorporation of ^{33}P. Read-out for phosphatase assay was absorbance at 410nm utilizing pNPP as substrate. The general kinase/phosphatase inhibitor staurosporine was used at 1µM as positive control. Further specific *in vitro* kinase assay methods may be found online at www.signalchem.com. An additional 451 kinases were tested by KINOME*Scan* (San Diego, CA) at 1µM LDK. Further information regarding KINOME*Scan* methods may be found online at www.kinomescan.com.

Cell cycle analysis

Cell cultures were suspended in media at a density of 500,000 cells/mL and exposed to the treatment conditions in 6-well cell culture plates for the indicated time at 37°C. Cells were harvested, washed with PBS, and fixed with 70% ethanol at 4°C for a minimum of 2 hours. Fixed cells were washed with PBS and stained with 10µg/mL propidium iodide (Invitrogen) with 1mg/mL RNase A (Invitrogen) for 30 minutes at 37°C, then placed on ice prior to analysis. DNA staining and cell cycle analysis was then done by flow cytometry.

LDK mouse blood serum level determination

Initial pharmacokinetic analysis was performed by Advinus, Inc. (Bangalore, India) (See Supplemental Fig. 7). Briefly, male Swiss Albino mice were divided into two groups and given a single dose bolus of LDK either intravenously at 3mg/kg via tail vein injection or orally at 15 mg/kg via gavage feeding. LDK solution was prepared by dissolving it in an aqueous diluent consisting of 5% N,N-dimethylacetamide, 5% Cremophor-EL, and 5% dextrose at pH 6.0 and filter-sterilizing it through a 0.22µm filter syringe. LDK for IV administration was resuspended in diluent to a concentration of 0.5 mg/mL with an injection volume of 6 mL/kg. LDK for oral administration was resuspended in the same diluent to a concentration of 1.5 mg/mL with an administration volume of 10 mL/kg. Following LDK administration, three treated mice were assessed per time point per treatment for blood serum LDK levels via retro-orbital blood draw and serum separation followed by LC-MS analysis. Pharmacokinetic parameters were calculated using non-compartmental analysis tool of WinNonlin Enterprise (Version 5.1.1) software (Pharsight Inc, Sunnyvale, CA). For LDK blood serum level determination following maximum tolerated dose study (See Supplemental Fig. 7), male NOD-SCID mice were divided into four groups of five mice each and injected IP b.i.d. for two weeks with either diluent only or 16, 80, or 200 mg/kg LDK, respectively. 24 hours following the last injection, mice were exsanguinated and serum was separated for LC/MS analysis of LDK serum levels by J.C.

Liquid chromatography-mass spectrometry (LC-MS)

For LC-MS analysis of LDK, an integrated system consisting of two Shimadzu LC-10AD VP pumps, a CBM-20A controller and an API 365 triple quadrupole mass spectrometer modified with an Ionics EP10+ source was employed. Parameter optimization was performed by direct infusion of LDK dissolved in acetonitrile. The parent mass of 287.2 m/z and the daughter mass of 144.2 was determined to be optimal for quantification in the positive mode. An HPLC method was developed using a HyperClone 100 x 4.6 mm 3 ODS column (Phenomenex, Torrance, CA). Solvents employed for this were MS grade acetonitrile and 5 mM ammonium formate buffer, pH 3.2. Flow rate was 0.5 mL/min. The column was held initially at 10% acetonitrile for 1 minute followed by a linear gradient to 90% acetonitrile over 4 minutes. This concentration was held for 2 additional minutes followed by a downward ramp back down to 10% acetonitrile. The column was re-equilibrated for 4 minutes at 10% acetonitrile. Using this method the retention time for LDK was determined to

be 6.3 minutes. A calibration curve was generated with the lower limit of detection (LOD) determined to be 6.25 ng/mL. A liquid-liquid extraction method was developed and optimized for the analysis of LDK in serum and tissue by spiking bovine serum with LDK and extracting twice with equal parts of toluene.

Isolation and analysis of mouse tissues

Groups of 3 animals per dose of drug were evaluated every 3 to 4 days by peripheral blood analysis and for cellularity and immunophenotype of cells in spleen and thymus. Peripheral blood was collected from the retro-orbital sinus and subjected to differential cell counting using a Serono System 9010+CP hematology counter (Serono Diagnostics, Allentown, PA). Spleen and thymus tissue was dissociated in Hank's Balanced Salt Solution (HBSS) containing 5% newborn calf serum, red blood cells were eliminated by ammonium chloride lysis. Cell counts were determined as above and 2 x 10^6 cells were incubated with labeled monoclonal antibodies to detect CD4, CD8, and CD19. Flow cytometry was performed using a FACScan flow cytometer (BD Biosciences, San Jose, CA; modified by Cytek Development, Fremont, CA). Four-micron thick sections were cut and stained with hematoxylin and eosin. Mouse tissues were examined for evidence of necrosis or toxicity by A.A.

B-ALL cell culture and treatment

Primary human B-ALL samples were cultured in StemSpan (Stem Cell Technologies, Vancouver, BC) serum-free expansion medium for hematopoietic cells supplemented with 1 mM L-glutamine, 10mM Hepes, 0.1mM non-essential amino acids, and 1mM sodium pyruvate. Cells were treated with test compounds or vehicle for 72 hours. During the last 16 hours of culture the cells were pulsed with [^{3}H]-thymidine. At the end of the incubation period the cells were harvested and run on a scintillation counter to measure dpm as a readout of cell proliferation.

CML clonogenic and viability analysis

Methylcellulose clonogenic assays were carried out by plating 10^3 CML-CP^{CD34+} or Ph+ALL MNCs 0.9% MethoCult (Stem Cell Technologies) in the presence of the cytokine cocktails described above, and either untreated or treated with LDK (0.1, 1.0, 3.0, 10.0μM) or imatinib (2.5μM). Colonies (>100μm) from primary cells were scored on day 12. Trypan blue exclusion was used to assess cell viability.

CD34+ hematopoetic stem/progenitor cell isolation and assay for LDK sensitivity

Umbilical cord blood samples were received within 48 hours of harvest. Samples were centrifuged and the pellet was suspended in red blood cell (RBC) lysis buffer and incubated on ice for 5 minutes. Cells were then pelleted, washed with PBS/2 mM EDTA, layered over cold Ficoll, and centrifuged at 1,800 RPM for 30 minutes. Mononuclear cells were then collected and washed in PBS/2 mM EDTA. CD34+ separation was then performed with an AutoMACS Pro separator and CD34 Multisort MicroBeads from Miltenyi Biotec (Auburn, CA). The CD34+ cells were then cultured in RPMI supplemented with 10% FBS, StemSpan® CC100 cytokine cocktail (STEMCELL Technologies, Vancouver, BC), and 50 ng/mL recombinant human thrombopoietin (Invitrogen, Carlsbad, CA). Cells were immediately plated in 96-well plates, treated with compound or vehicle, and incubated for 48 hours. After incubation the cell viability was determined by MTT analysis.

Supplemental Reference

1. Hahn M, Li W, Yu C, Rahmani M, Dent P, Grant S. Rapamycin and UCN-01 synergistically induce apoptosis in human leukemia cells through a process that is regulated by the Raf-1/MEK/ERK, Akt, and JNK signal transduction pathways. *Mol Cancer Ther*. 2005**;** 4(3):457-70.